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PEPTIDES—XXXXIV

SYNTHESIS OF THE 105–117 FRAGMENT OF A LYSOZYME ANALOGUE

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Abstract—The synthesis of the (105–117) fragment of a Lysozyme analogue is described. This sequence was assembled by DCCI/HONSu fragment coupling of the (105–111) and (112–117) subfragments which were both constructed by the fragment coupling method. The arginine residue at position 114 was initially unprotected but ultimately protection was afforded by the use of the adamantyloxycarbonyl group.

The (105–117) fragment of the lysozyme analogue[1] which is the subject of our programme of synthesis, contains an arginine residue at position-114. This arginine residue was considered to be essential to lysozyme activity since it is known to have a role in substrate binding,[2] and therefore could not be omitted from the analogue sequence. All the other arginine residues were replaced by ornithine. The amino acid sequence of the (105–117) fragment is given below.

Nle. Asn. Ala. Trp. Val. Ala. Trp.
105 108 111

Orn. Asn. Arg. Cys. Lys. Gly.
112 114

The norleucine residue-105 has replaced methionine in order to simplify the synthetic procedures and to provide a diagnostic ratio for assessing coupling efficiency and purity of products in a similar way to that which was used earlier in the 1–16 fragment at residue-12.[3] From the sequence it is clear that there are no points at which racemisation free coupling could be carried out. Therefore, it was decided to construct the molecule by fragment coupling involving hepta- and hexapeptides between tryptophan-111 and ornithine-112 as this would allow the maximum use of gel filtration chromatography for purification. Both the constituent peptides were themselves assembled by a fragment condensation procedure; the heptapeptide by a 4+3 fragment condensation and the hexapeptide by a 3+3 coupling.

The first synthesis of the (112–114) tripeptide was carried out with the arginine side-chain unprotected (Scheme 1) with the intention of producing intermediates with a polar handle which could be purified by counter current distribution. The dipeptide (**123**)* was prepared by active ester coupling using the appropriate asparagine *N*-hydroxysuccinimide active ester.[4] The protected dipeptide acid (**123**) was deprotected by treatment with HBr in glacial acetic acid. This led to the free dipeptide dihydrobromide which was then condensed with the ornithine active ester (**11**).[3] This coupling gave a high yield of the crude tripeptide (**124**) (88%), which was eventually purified by counter current distribution giving a final yield of 55%.

The next stage in the synthesis involved coupling of this fragment to the (115–117) tripeptide. Initial trial experiments using the DCCI/HONSu[5] coupling method showed that many side products were produced and purification by counter current distribution was found to be extremely difficult. Following this finding it was

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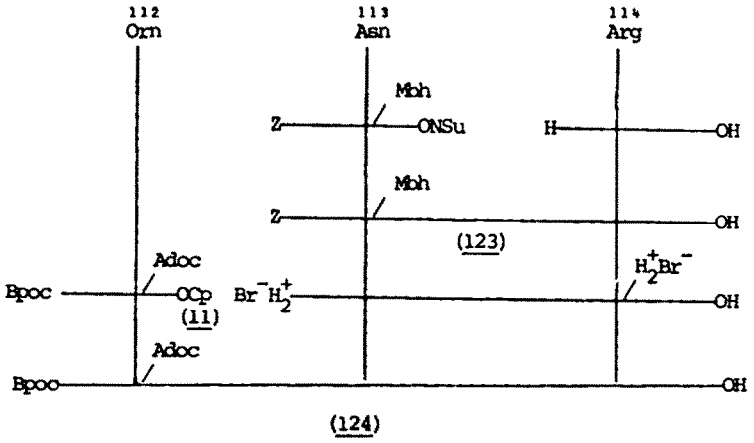
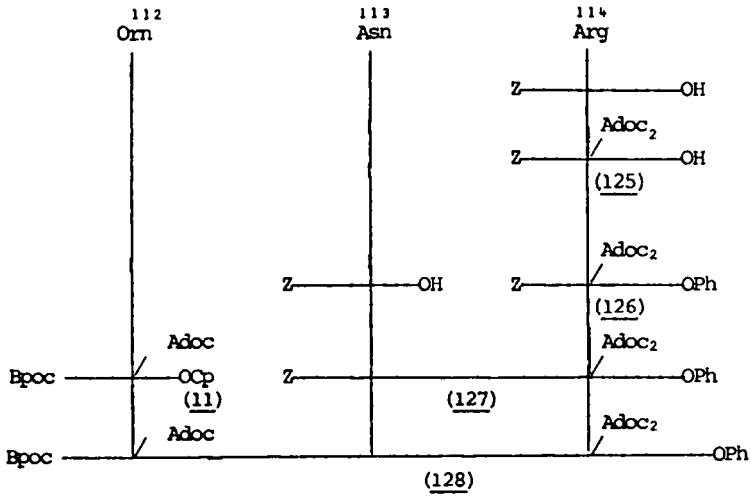


Scheme 1. First route to the (112–114) tripeptide.

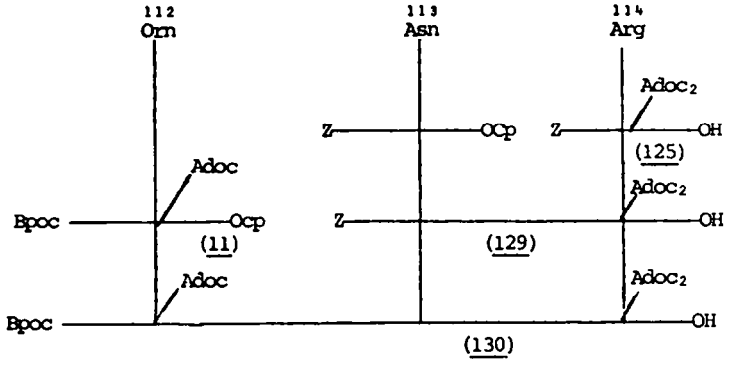


Scheme 2. Second route to the (112–114) tripeptide.

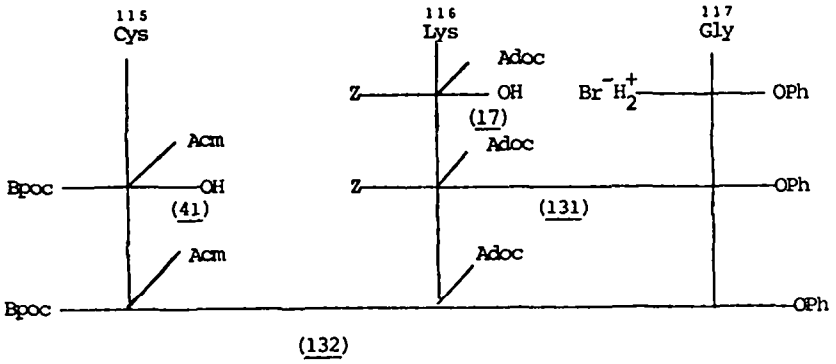
decided to abandon the route in favour of one involving complete protection of the arginine residue (Scheme 2), accepting that this would necessitate changing from counter current distribution to gel filtration as the main means of purification. In order to maintain consistency with other side-chain protection used in the synthesis the adamantyloxycarbonyl group was utilised for the protection of the guanidine function of arginine in the form of $N^{G}N^{\omega}$-bis adamantyloxycarbonylarginine.[6] Although the compound (**125**) is a known compound we experienced considerable difficulty in its preparation. Firstly, the benzyloxycarbonylarginine used for the preparation of this derivative must be absolutely pure and preparation is best achieved using careful pH control maintaining the pH at 9.4 during the introduction of the benzyloxycarbonyl function. Four equivalents of freshly prepared adamantylchloroformate[7] were used in the preparation of the derivative (**125**) and providing the work up procedure is rigorously adhered to a yield of derivative in the 50–60% region was generally attained. Reaction of the derivative (**125**) with phenol in the presence of DCCI and pyridine gave an 80% yield of the corresponding phenyl ester (**126**). The N^{α}-protection was then removed by hydrogenolysis in the presence of *p*-toluenesulphonic acid and the resulting salt was coupled with Z.Asn.OH by the pivaloyl mixed anhydride method to afford the fully protected dipeptide (**127**). Hydrogenolysis of this dipeptide and subsequent coupling with the ornithine active ester (**11**)[3] in the presence of hydroxybenzotriazole[8] led to the crude fully protected tripeptide (**128**). Adequate purification could not be achieved using gel filtration on Sephadex LH20 although the product from this chromatography was clearly mainly the required protected tripeptide (**128**).

The alternative of a salt coupling approach was then investigated (Scheme 3) in which case the N^{α}-protection was removed from the derivative (**125**) by hydrogenolysis in 90% glacial acetic acid. This gave the appropriate protected arginine zwitterion which could be coupled with Z.Asn.OCp,[9] in this case one equivalent of HOBt being added to catalyse the reaction. The resulting dipeptide (**129**) was readily purified and the benzyloxycarbonyl function was easily removed by hydrogenolysis using a mixture of DMF and 90% acetic acid (1:1) giving complete cleavage in 2 hr. If DMF alone were used as a solvent for the hydrogenolysis the reaction was found to be incomplete even after 8 hr. Coupling with the ornithine trichlorophenyl active ester (**11**)[3] was then carried out, again in the presence of HOBt. The tripeptide (**130**) was purified effectively by gel filtration in a yield of 78%, thus a salt coupling approach was that selected for the final synthesis.

The (115–117) tripeptide fragment was assembled by the route shown (Scheme 4). The dipeptide (**131**) being



Scheme 3. Third route to the (112–114) tripeptide.

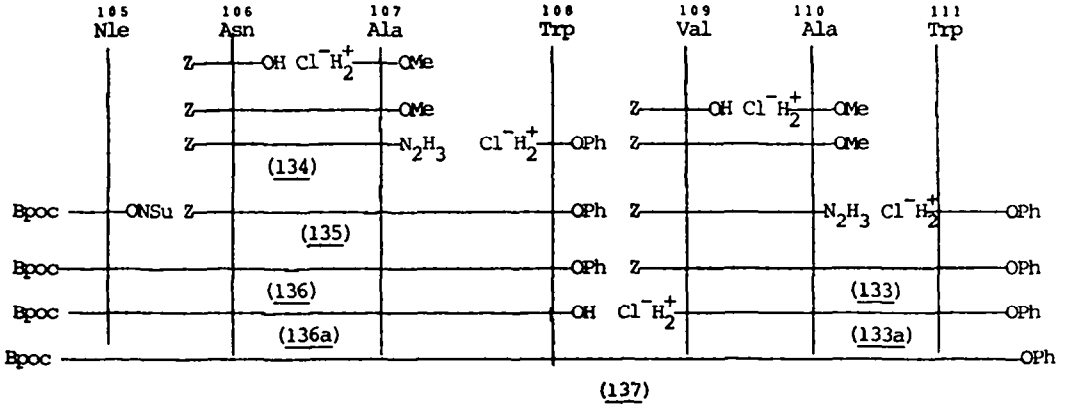


Scheme 4. Synthesis of the protected (115–117) fragment (132).

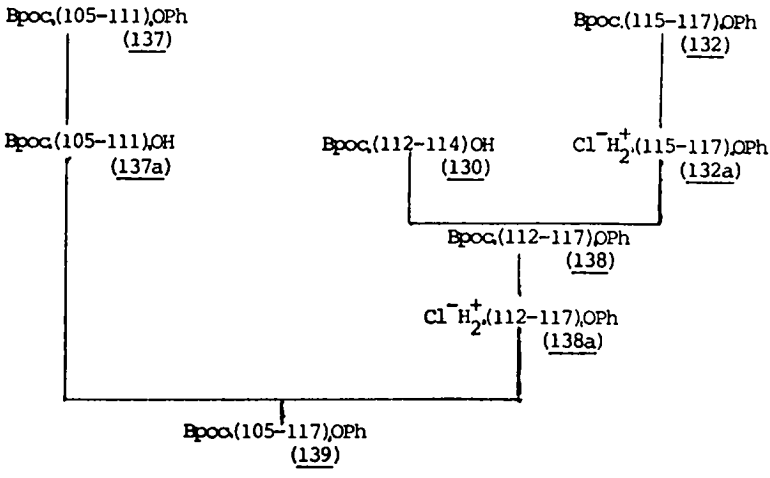
formed by pivalic mixed anhydride coupling between the lysine derivative (17)[3] and glycine phenyl ester hydrobromide.[10] The amino-protecting group was removed from the dipeptide (131) by hydrogenolysis in the usual manner. The resulting salt was coupled to the cysteine derivative (41)[11] once again using the pivalic mixed anhydride method. Purification of the fully protected tripeptide (132) was best achieved using gel filtration on Sephadex LH20 eluting with DMF, which gave the required tripeptide (132) in pure form in a yield of 80%.

The synthesis of the (105–111) heptapeptide was then embarked upon using the 4+3 fragment condensation approach (Scheme 5). Z.Val.Ala.N_2H_3[12] was converted to the corresponding azide and coupled with tryptophan phenyl ester hydrochloride,[10] giving the tripeptide (133) in 85% yield. The (105–108) tetrapeptide assembly also involved the use of the azide method, thus Z.Asn.Ala.OMe[13] was converted to the corresponding hydrazide (134) in almost quantitative yield by treatment with hydrazine hydrate. This hydrazide being converted to the corresponding azide prior to coupling with tryptophan phenyl ester hydrochloride[10] to give the protected tripeptide (135). After hydrogenolysis of the N^{α}-amino protecting group, the norleucine residue was added using the hydroxysuccinimide ester shown in Scheme 5. This gave the required tetrapeptide (136) in quantitative yield. The protected tripeptide (133) was hydrogenolysed in DMF in the presence of 5% Pd/C and HCl as use of 10% Pd/C for this reaction gave an inferior product, probably due to side reactions involving tryptophan. The tetrapeptide acid (136a) was prepared by phenyl ester cleavage of the ester (136) using our standard conditions.[10] The cleavage which was complete in 30 min gave an 80% yield of the tetrapeptide acid (136a). Coupling of these two components was then carried out by the DCCI/HONSu method, the reaction being allowed to proceed for 3 days. The required heptapeptide (137) was then isolated after gel filtration on Sephadex LH20, being obtained in 60% yield.

The final assembly of the (105–117) fragment was then carried out by the route shown (Scheme 6). The (112–117) protected hexapeptide (138) was initially assembled from the tripeptides (130) and (132a). The tripeptide (132) was converted to its corresponding hydrochloride (132a) by treatment with HCl in dichloromethane. The cleavage of a Bpoc group from *N*-terminal cysteine appears to be particularly facile which we attribute to intramolecular participation in the cleavage by the sulphur atom in the cysteine side-chain. The resulting hydrochloride (132a) was homogeneous and was used directly in coupling with the (112–114) tripeptide acid (130). On this occasion the coupling was carried out using the DCCI/HONSu method over a period of 3 days. The required hexapeptide (138) was isolated after LH20 chromatography in 69% yield. The Bpoc protecting group was then removed from the protected hexapeptide (138), whilst maintaining the pH at 0.5. These conditions gave rapid cleavage of the protecting group producing the peptide hydrochloride (138a) in high yield within 35 min. The heptapeptide acid (137a) was prepared by carrying out ester cleavage on the protected heptapeptide phenyl ester (137). Cleavage using DMF/H_2O (1:2) as the solvent was complete



Scheme 5. Synthesis of the protected (105–111) fragment (137).



Scheme 6. Synthesis of the fully protected (105–117) fragment (**139**).

within 1 hr when the pH was maintained at 10.5, DMF and hydrogen peroxide being added in accordance with our general cleavage conditions.[10]

Coupling of the two fragments (**137a**) and (**138a**) by the DCCI/HONSu method was effected using DMF as solvent and NMM as base. A second addition of DCCI and HONSu being made after 24 hr. After a total reaction time of 3 days the reaction mixture was filtered and applied directly to Sephadex LH20 and eluted with DMF. The tridecapeptide (**139**) was isolated by pooling the appropriate fractions from the peak with a (Ve/Vt) ratio of 0.37, rechromatography being carried out in order to obtain absolutely pure material which could be used subsequently for the assembly of larger fragments. The protected (105–117) fragment thus obtained was homogeneous in three TLC systems and by electrophoresis of the completely deprotected material at pH 2.1. The amino acid analysis of the fragment was satisfactory showing the diagnostic amino acids arginine and norleucine to be present in their correct ratios.

EXPERIMENTAL

The general experimental methods, abbreviations, and TLC systems are those described in earlier papers in this series.

Z-Asn(Mbh)-Arg-OH (**123**). A solution containing $Cl^-H_2^+$.Arg.OH (17.6 g, 84 mM) in 1M NaOH (84 ml) was slowly added to a solution containing Z-Asn(Mbh)-ONSu in DMF (350 ml). After stirring for 4 days the resulting suspension was poured into H_2O (3.5 l) and the precipitated product filtered, washed with water and dried giving the required product (**123**) (42.8 g, 77%), m.p. 184–185°, $[\alpha]_D^{25}$ + 1.5° (c = 1.5, DMF), $R_f(9)^-$0.2, $Arg_{1.00}Asp_{1.00}$, (Found: C, 59.61; H, 6.42; N, 12.35; $C_{33}H_{40}N_6O_8$. H_2O requires: C, 59.45; H, 6.35; N, 12.60%).

Bpoc-Orn(Adoc)-Asn-Arg-OH (**124**). Z-Asn(Mbh)-Arg-OH (**123**) (6.45 g, 10 mM) was dissolved in glacial HOAc (150 ml) and anisole (12 ml) and HBr in glacial HOAc (45% w/v, 80 ml) added. After 6 hr at room temperature Et_2O was added to precipitate the product, trituration with Et_2O and drying gave the hygroscopic dihydrobromide (4.74 g, 100%), $R_f(23)$–0.1. This hydrobromide, NMM (2.2 ml, 20 mM) and pyridine (1.6 ml, 20 mM) were dissolved in DMF (60 ml) followed by the active ester (**11**) (7.64 g, 10.5 mM) and the reaction mixture stirred for 3 days. *N,N*-Dimethylaminopropylamine (1.26 ml, 10.5 mM) was added, and after 30 min the reaction concentrated and poured into ice cold citric acid (400 ml). The aqueous solution was washed with Et_2O then extracted into $CHCl_3$; evaporation of this solution gave a yellow oil which was dissolved in MeOH (20 ml) and precipitated with Et_2O (500 ml). The product was filtered, washed with Et_2O and dried giving the crude tripeptide (**124**) (7.1 g, 88%). Purification was achieved by counter current distribution in $CHCl_3/CCl_4$/MeOH/0.2 M aq.NH_4OAc (pH7); 7:3:10:4, 350 transfers, K = 0.69. The purified material was precipitated by addition of Et_2O after evaporation giving (**124**) (4.4 g, 55%) m.p. 152°, $[\alpha]_d^{20}$ + 1.4° (c = 2, DMF), $R_f(23)$–0.5, $R_f(28)$–0.8, $Orn_{1.03}Arg_{0.97}$ $Asp_{1.00}$, (Found: C, 58.43; H, 7.38; N, 13.42; $C_{42}H_{58}N_8O_9$.2.5H_2O requires: C, 58.39; H, 7.35; N, 12.97%).

Z-Arg(Adoc)$_2$-OH (**125**). A solution containing Z-Arg-OH (20.7 g, 62.7 mM) in dioxan (40 ml) and 2M NaOH (134 ml) was cooled to 6–8°. Freshly prepared Adoc.Cl (57.4 g, 268 mM) in dioxan (50 ml) and 2M NaOH (200 ml) were then consecutively added over a period of 1 hr and the reaction maintained at 6–8° for 2 hr. The mixture was centrifuged and the residue triturated and washed with Et_2O. The Et_2O washings were evaporated and the residue triturated with petroleum ether (60–80°), filtered, washed with petroleum ether and recombined with the original residue. The combined residues were again washed with petroleum ether and then suspended in H_2O prior to acidification with 0.5 M citric acid (pH 2.5). The resulting suspension was extracted into Et_2O and evaporated to yield a residue which was crystallised from MeOH/H_2O giving the required derivative (**125**) (22.0 g, 53%), m.p. 120°, $[\alpha]_D^{20}$ – 0.8° (c = 1, DMF), $R_f(2)$ – 0.2, $R_f(23)$ – 0.9, (Found : C, 63.25; H, 7.28; N, 8.30; $C_{36}H_{48}N_4O_8$.H_2O requires: C, 63.32; H, 7.38; N, 8.21%); lit.[5] m.p. 120–122° dec; $[\alpha]_D^{20}$ + 20.8° (c = 1, $CHCl_3$).

Z-Arg(Adoc)$_2$-OPh (**126**). The protected amino-acid (**125**) (1.0 g, 1.5 mM) and phenol (0.15 g, 1.58 mM) were dissolved in EtOAc (4 ml) and cooled to −5°. Pyridine (0.12 ml, 1.5 mM) and DCCI (0.37 g, 1.80 mM) were dissolved in EtOAc (0.5 ml) and added to the cooled solution. After 18 hr the DCU was removed by filtration, and the filtrate evaporated to given an oil which was crystallised from IPA/H_2O yielding (**126**) (0.9 g, 80%), m.p. 75–77°, $[\alpha]_D^{20}$ – 6.5° (c = 1, DMF), $R_f(19)$ – 0.7, $R_f(26)$ – 0.9, (Found: C, 67.54; H, 7.31; N, 7.78; $C_{42}H_{52}N_4O_8$0.5 H_2O requires: C, 67.27; H, 7.12; N, 7.47%).

Z-Asn-Arg(Adoc)$_2$-OPh (**127**). The preceding derivative (**126**) (0.32 g, 0.5 mM) and Tos.OH.H_2O (0.1 g, 0.5 mM) were dissolved in DMF (4 ml) and hydrogenolysed for 6 hr in the presence of 10% Pd/C (25 mg). Work up in the usual way gave the salt as a gum, $R_f(2)$ – 0.4. Z-Asn-OH was dissolved in DMF (2 ml) and the solution cooled to −20°. NMM (0.7 ml, 0.6 mM) and pyridine (0.5 ml, 0.58 mM) were added, followed by Piv.Cl (0.7 g, 0.58 mM) in DMF (0.5 ml). After 20 min the salt from the hydrogenolysis above was dissolved in DMF (1 ml) and NMM (0.6 ml, 0.5 mM) added, this solution being added to the mixed anhydride previously prepared. After 2 hr at −20° and 18 hr at room temperature the reaction mixture was evaporated and 2M $NaHCO_3$ added to precipitate the product. This was washed with 1M HOAc and 2M $NaHCO_3$ alternately, then finally with water. The dried solid was reprecipitated from IPA/H_2O giving (**127**) (0.13 g,

30%) m.p. 139–142°, $[\alpha]_D^{24}$ – 11.8° (c = 1, DMF), $R_f(25)$ – 0.6, $R_f(8)$ – 0.3, $Arg_{0.93}Asp_{1.07}$, (Found: C, 63.36; H, 7.18; N, 9.96; $C_{46}H_{58}N_6O_{16}$. H_2O requires: C, 63.29; H, 6.93; N, 9.69%).

Bpoc–Orn(Adoc)–Asn–Arg(Adoc)$_2$–OPh (**128**). The protected dipeptide (**127**) (96 mg, 0.11 mM) and Tos.OH.H_2O (21 mg, 0.11 mM) were dissolved in DMF (4 ml) and hydrogenolysed for 8 hr in the presence of 10% Pd/C (6 mg). The usual work up gave a residue $R_f(25)$ – 0.3 which was dissolved in DMF (1 ml) along with the active ester (**11**) (60 mg, 0.11 mM), HOBt (15 mg, 0.11 mM) and NMM (0.013 ml, 0.11 mM). After 18 hr the solvent was evaporated and the residue partitioned between EtOAc and H_2O. The organic layer being washed with 2M $NaHCO_3$, 5% citric acid and water, then dried and evaporated. The resulting gum was subjected to purification on Sephadex LH20 eluting with DMF (Ve/Vt) = 0.43, the material isolated (62 mg, 45%), $R_f(25)$ – 0.5 (major) and 0.6 appeared to be mainly the required (**128**) by NMR but was clearly not homogeneous and did not give an acceptable amino acid analysis. Further purification of the product was not successful.

Z–Asn–Arg(Adoc)$_2$–OH (**129**). The arginine derivative (**125**) (19.9 g, 30 mM) was dissolved in aq. 90% HOAc (70 ml) and hydrogenolysed for 3 hr in the presence of 10% Pd/C (1.51 g). Filtration and evaporation of the filtrate gave a product which was crystallised from MeOH/Et_2O giving (12.0 g, 75%), $R_f(2)$ – 0.3. This zwitterion, (2.2 g, 4.1 mM), Z–Asn–OCp (1.8 g, 4.0 mM), TEA (0.56 ml, 4 mM) and HOBt (0.5 g, 4 mM) were dissolved in DMF (20 ml) and stirred for 18 hr. Evaporation gave a residue to which 2M $NaHCO_3$ was added, the resulting precipitate was washed with Et_2O and ice-cold 5% citric acid then again washed with water and Et_2O. Crystallisation from EtOAc/petroleum ether gave the crude dipeptide (**129**) which was further purified by silica gel chromatography eluting with $CHCl_3$/MeOH (9:1) yielding the purified material (1.8 g, 57%), m.p. 142–144°, $[\alpha]_D^{20}$ + 2.2° (c = 1, DMF), $R_f(2)$ – 0.3, $R_f(21)$ – 0.5, $Arg_{1.03}Asp_{1.00}$, (Found: C, 60.71; H, 7.09; N, 10.59; $C_{40}H_{54}N_6O_{10}$. 0.5 H_2O requires: C, 60.98; H, 7.04; N, 10.67%).

Bpoc–Orn(Adoc)–Asn–Arg(Adoc)$_2$–OH. (**130**). The protected dipeptide (**129**) (4.4 g, 5.6 mM) was dissolved in DMF (8 ml) and 90% HOAc (8 ml) and hydrogenolysed for 2 hr in the presence of 10% Pd/C (0.28 g). Work up in the usual way gave a residue which was dissolved in DMF (18 ml); the ornithine active ester (**11**) (3.6 g, 5 mM), HOBt (0.7 g, 5 mM) and TEA (0.7 ml, 5 mM) were added and the solution stirred for 18 hr. The reaction mixture was then poured into 2M $NaHCO_3$ and the precipitate filtered. This material was partitioned between EtOAc and ice cold 5% citric acid, the organic phase was washed with water and brine then evaporated to yield a residue which was dissolved in DMF and chromatographed on Sephadex LH20 and eluted with DMF. The required tripeptide (**130**) eluted with (Ve/Vt) = 0.44 and isolation gave (4.6 g, 78%), m.p. 145–146°, $[\alpha]_D^{20}$ + 1.5° (c = 1, DMF), $R_f(2)$ – 0.1, $R_f(17)$ – 0.4, $Orn_{0.98}Arg_{1.00}Asp_{1.00}$, (Found: C, 63.31; H, 7.54; N, 9.06; $C_{64}H_{86}N_8O_{13}$. $2H_2O$ requires: C, 63.45; H, 7.49; N, 9.25%).

Z–Lys(Adoc)–Gly–OPh (**131**). Z–Lys(Adoc)–OH (**17**) (39.0 g, 85 mM) was dissolved in DCM (180 ml) and cooled to – 10°. NMM (9.4 ml, 85 mM) and pivaloyl chloride (10.1 g, 84 mM) were consecutively added and 20 min allowed for activation. A DMF (70 ml) solution containing $Br^-H_2^+$–Gly–OPh (21.1 g, 91 mM) and NMM (10.0 ml, 91 mM) was cooled and added then the reaction mixture permitted to reach room temperature overnight. The solution was evaporated and the residue dissolved in EtOAc, this solution being washed with 0.1M NaOH, 5% citric acid, water and brine. This solution was evaporated to give a residue which was crystallised from EtOAc/cyclohexane yielding (**131**) (35.2 g, 70%), m.p. 119–121°, $R_f(7)$ – 0.8, $R_f(9)$ – 0.7, $[\alpha]_D$ – 15.6° (c = 1, DMF), $Lys_{0.95}Gly_{1.00}$, (Found: C, 66.72; H, 6.87; N, 7.01; $C_{33}H_{41}N_3O_7$ requires: C, 66.99; H, 6.98; N, 7.10%).

Bpoc–Cys(Acm)–Lys(Adoc)–Gly–OPh (**132**). The dipeptide (**131**) (5.9 g, 10 mM) and Tos.OH.H_2O (1.9 g, 10 mM) were dissolved in DMF (50 ml) and hydrogenolysed for 5 hr in the presence of 10% Pd/C (0.5 g). The reaction mixture was filtered and the solution volume reduced to 15 ml. Bpoc–Cys(Acm)–OH (**41**) (5.2 g, 10 mM) was dissolved in DMF (15 ml) and a solution of NMM (1.1 ml, 10 mM) in DMF (3 ml) added. After cooling to –20° pivaloyl chloride (1.4 ml, 10 mM) was added and 20 min allowed for activation. The solution of the amino-component was added followed by NMM (1.1 ml, 10 mM) and the solution stirred, gradually warming to room temperature overnight. Evaporation gave a residue which was partitioned between EtOAc and H_2O. The solution was washed with 2M $NaHCO_3$, 5% citric acid, water and brine then dried and evaporated. The residue was dissolved in DMF and chromatographed on Sephadex LH20 eluting with DMF. The product eluted with (Ve/Vt) = 0.52 and on work up gave (**132**) (6.9 g, 80%), m.p. 90–93°; $[\alpha]_D^{20}$ – 22.0° (c = 0.75, DMF), $R_f(25)$ – 0.4, $R_f(21)$ – 0.3, $Lys_{0.95}Gly_{1.05}$, (Found: C, 64.62; H, 7.09, N, 7.88; $C_{47}H_{59}N_5O_9S$ requires: C, 64.88; H, 6.83; N, 8.05%).

Z–Val–Ala–Trp–OPh (**133**). Z–Val–Ala–N_2N_3[11] (16.8 g, 50 mM) was suspended in DMF (125 ml) and after cooling to –20° HCl in dioxan (4.92M, 40.65 ml, 200 mM) added. Freshly distilled *tert*-butyl nitrite (5.75 ml, 50 mM) was added and after 10 min the solution was cooled to –60° and TEA (28.0 ml, 200 mM) added, followed by a slurry of $Cl^-H_2^+$–Trp–OPh (15.8 g, 50 mM) in DMF (70 ml) and additional TEA (7.0 ml, 50 mM). The solution was warmed to –20° and maintained at this temperature for 1 hr then kept at –10° for 70 hr. The reaction mixture was then poured into rapidly stirred H_2O (21) and the resulting suspension filtered and washed with 2M $NaHCO_3$, 5% citric acid and water. Reprecipitation from DMF/EtOH gave (**133**) (24.8 g, 85%), m.p. 210–212°, $[\alpha]_D^{25}$ – 1.6° (c = 1.5, DMF), $R_f(2)$ – 0.8, $Ala_{0.97}Val_{1.03}$, (Found: C, 67.50; H, 6.24; N, 9.51; $C_{33}H_{36}N_4O_6$ requires: C, 67.79; H, 6.21; N, 9.58%).

Z–Asn–Ala–N_2H_3 (**134**). Z–Asn–Ala–OMe[12] (22.5 g, 64 mM) was dissolved in warm DMF (500 ml), hydrazine hydrate (32 ml, 640 mM) added and the solution stirred at room temperature for 18 hr. The resulting suspension was concentrated to approximately (100 ml) and EtOH (600 ml) added. After cooling the thick suspension was filtered giving the required hydrazide (**134**). (21.9 g, 97%), m.p. 220–223°, $[\alpha]_D$ too insoluble for measurement, $R_f(2)$ – 0.2, (Found: C, 51.25; H, 6.05; N, 20.06; $C_{15}H_{21}N_5O_5$ requires: C, 51.28; H, 6.02; N, 19.93%).

Z–Asn–Ala–Trp–OPh (**135**). The hydrazide (**134**) (17.5 g, 50 mM) was suspended in DMF (300 ml) and cooled to –20°. A solution of HCl in dioxan (4.92M, 40.6 ml, 200 mM) was added and stirred until the hydrazide had dissolved (approx. 5 min). Freshly distilled *tert*-butyl nitrite (5.75 ml, 50 mM) was added and after 10 min the solution was cooled to –60°. TEA (28.0 ml, 200 mM) was added followed by $Cl^-H_2^+$–Trp–OPh (15.8 g, 50 mM) in DMF (70 ml). A further portion of TEA (7.0 ml, 50 mM) was added and the reaction mixture stirred for 3 days at –10°. The resulting suspension was poured into water and the precipitated product filtered, washed with 2M $NaHCO_3$, 5% citric acid and water. After further washings the product was dried giving (**135**) (27.7 g, 92%), m.p. 199–203°, $[\alpha]_D^{25}$ – 9.9° (c = 1.5, DMF), $R_f(2)$ – 0.2, Amino acid analysis (3M Tos.OH, tryptamine, 108°.) $Trp_{0.97}Asp_{1.00}Ala_{1.02}$, (Found: C, 62.38; H, 5.58; N, 11.14; $C_{32}H_{33}N_5O_7.H_2O$ requires: C, 62.23; H, 5.71; N, 11.34%).

Bpoc–Nle–Asn–Ala–Trp–OPh (**136**). The protected tripeptide (**135**) (15.0 g, 24 mM) was dissolved in DMF (150 ml) and 5% Pd/C (2.5 g) added. A solution of HCl in EtOAc (3.3M, 8 ml, 26.4 mM) was added and hydrogenolysis carried out for 3.5 hr. Filtration and evaporation of the filtrate gave an off-white solid (11.6 g, 96%). This solid (1.0 g, 2 mM) and NMM (202 mg, 2 mM) were dissolved in DMF (2 ml). After cooling to 0° Bpoc–Nle–ONSu (1.07 g, 2.3 mM) was added and the solution stirred at room temperature for 3 days. The reaction mixture was evaporated and the residue dissolved in EtOAc, Et_2O being added to give the required product (**136**) (1.6 g, 95%), m.p. 122–123°, $[\alpha]_D^{26}$ – 18.0° (c = 1.5, DMF), $R_f(3)$ – 0.8, $Asp_{1.00}Ala_{0.98}Nle_{1.00}$, (Found: C, 65.98; H, 6.61; N, 10.28; $C_{46}H_{52}N_6O_8.H_2O$ requires: C, 66.17; H, 6.52; N, 10.06%).

Bpoc(105–111).OPh (**137**). (a) $Cl^-H_2^+$.(109–111).OPh (**133a**). The protected tripeptide (**133**) (2.3 g, 4 mM) was dissolved in DMF (30 ml) and 5% Pd/C (200 mg) added. A solution of HCl in dioxan (4.92M, 0.88 ml, 4.32 mM) was added and the solution hydrogenolysed for 3 hr. Filtration and evaporation of the filtrate gave a residue which was reprecipitated from MeOH/Et_2O yielding the salt (**133a**) (1.77 g, 88%), m.p. 208–212°, $[\alpha]_D^{27}$ + 6.3° (c =

1.5, DMF), $R_f(7)-0.4$, (Found: C, 59.80; H, 6.66; N, 10.96; $C_{25}H_{31}N_4O_4Cl.H_2O$ requires: C, 59.46; H, 6.59; N, 11.09%).

(b) Bpoc(105–108).OH (**136a**). The peptide phenyl ester (12.9 g, 16.5 mM) was dissolved in DMF (150 ml) and water (50 ml) slowly added in a way such as to avoid precipitation of the ester. DMS (36 ml) was added, followed by 100 vol.H_2O_2 (1.6 ml, 16 mM). The pH of the solution was adjusted to 10.5 with 2M NaOH and maintained at this pH for 30 min. During this period 100 vol.H_2O_2 (1.6 ml, 16 mM) was twice added. The pH was then brought to 7 with ice-cold 5% citric acid and the solution volume reduced to approx. 50 ml. Further 5% citric acid was added to bring the pH to 4 and brine added to precipitate the product, filtration and drying gave the required peptide acid (**136a**) (9.6 g, 80%), m.p. 120°, $[\alpha]_D^{26}+16.7°$, $R_f(28)-0.7$, $R_f(3)-0.7$, (Found: C, 63.30; H, 7.25; N, 11.09; $C_{40}H_{48}N_6O_8.H_2O$ requires: C, 63.31; H, 6.64; N, 11.07%).

(c) *Coupling.* Bpoc(105–108).OH (**136a**) (9.3 g, 12.3 mM), $Cl^-H_2^+$ (109–111)OPh (**133a**) (6.1 g, 12.1 mM), HONSu (2.9 g, 25 mM) and NMM (1.3 g, 12.5 mM) were dissolved in DMF (60 ml) and the solution cooled to −15°, DCCI (2.8 g, 13.5 mM) was added and the solution stirred at room temperature for 3 days. The reaction mixture was filtered and the filtrate concentrated to approx. 40 ml. The product was precipitated by the addition of brine, the crude product was dried and dissolved in DMF then purified by gel filtration on Sephadex LH20 eluting with DMF. The purified product with (Ve/Vt) = 0.42, was precipitated by the addition of water to the concentrated fractions giving (**137**) (8.65 g, 60%), m.p. 300°, $[\alpha]_D^{20}+28.0°$ (c = 1, DMF), $R_f(14)-0.8$, $Asp_{1.01}Ala_{1.93}Val_{1.00}Nle_{1.01}$ (Found: C, 65.25; H, 6.50; N, 11.54; $C_{65}H_{76}N_{10}O_{11}.H_2O$ requires: C, 65.52; H, 6.60; N, 11.76%).

Bpoc(112–117)OPh (**138**). The protected tripeptide (**132**) (7.0 g, 0.8 mM) in DCM (135 ml) was treated with HCl in DCM (3.78 ml, 5.45M). After 30 min the solvent was evaporated and the resulting residue dissolved in DCM and added dropwise to Et_2O. Filtration gave (**132a**) (4.7 g, 88%), $R_f(2)-0.4$. The tripeptide acid (**130**) (1.18 g, 1.0 mM) and the hydrochloride (**132a**) (0.67 g, 1.0 mM) were dissolved in DMF (10 ml). NMM (0.11 ml, 1.0 mM) and HONSu (0.23 g, 2 mM) were added and the solution cooled to −5° prior to the addition of DCCI (0.28 g, 1.25 mM). After 24 hr at room temperature the solution was recooled and further portions of HONSu (0.115 g, 1.0 mM) and DCCI (0.14 g, 0.625 mM) added. The reaction was stirred for 3 days then filtered and the filtrate applied directly to Sephadex LH20 being eluted with DMF. The product having (Ve/Vt) = 0.41 was precipitated after concentration of the appropriate fractions by the addition of H_2O yielding (**138**) (1.25 g, 69%), m.p. 138°, $[\alpha]_D^{20}-13.8°$ (c = 1, DMF), $R_f(11)0.6$, $R_f(17)-0.7$ $Lys/Orn_{1.90}Arg_{1.02}Asp_{0.99}Gly_{1.06}$, (Found: C, 62.13; H, 7.39; N, 9.96; $C_{95}H_{129}N_{13}O_{19}S.3H_2O$ requires: C, 61.90; H, 7.38; N, 9.88%).

Bpoc(105–117)OPh (**139**). (a) $Cl^-H_2^+$(112–117)OPh (**138a**). The protected hexapeptide (**138**) (2.0 g, 1.12 mM) and DMS (4.1 ml, 56 mM) were dissolved in TFE (9 ml) and H_2O (1 ml) added. The pH was adjusted to 0.5 with 0.1M HCl in TFE/H_2O (9:1) and maintained at this value for 35 min. The solvent was evaporated and the hydrochloride (**138a**) precipitated with Et_2O giving (1.7 g, 95%), $R_f(17)-0.6$.

(b) Bpoc(105–111)OH (**137a**). Bpoc(105–111)OPh (**137**) (1.0 g, 0.83 mM) was dissolved in DMF (8 ml), DMF/H_2O (1:2) (6 ml) and DMS (3.0 ml, 41.5 mM) were added and the pH brought to 10.5 using 0.5M NaOH. 100 vol.H_2O_2 (0.083 ml, 0.83 mM) was added and the pH maintained at 10.5 for 1 hr. The pH was reduced to 4.0 with 5% citric acid and the product precipitated by pouring into H_2O. Filtration and drying gave (**137a**) (0.83 g, 92%), m.p. 224°, $[\alpha]_D^{20}-24.8°$ (c = 1, DMF), $R_f(11)-0.3$, (Found: C, 62.23; H, 6.98; N, 12.48; $C_{59}H_{72}N_{10}O_{11}.2H_2O$ requires: C, 62.53; H, 6.76; N, 12.36%).

(c) *Coupling.* The peptide acid (**137a**) (1.1 g, 1.0 mM) and the hydrochloride (**138a**) (1.59 g, 1.0 mM) were dissolved in DMF together with NMM (0.11 ml, 1.0 mM) and HONSu (0.23 g, 2 mM). After cooling to −5° DCCI (0.28 g, 1.25 mM) was added and the solution stirred at room temperature overnight. The solution was recooled and further portions of HONSu (0.115 g) and DCCI (0.14 g) were added. After allowing 3 days for completion of the reaction the reaction mixture was applied directly to Sephadex LH20 being eluted with DMF. The product with (Ve/Vt) = 0.36 was isolated by evaporation and precipitation with water giving Bpoc(105–117)OPh (**139**) (1.5 g, 57%), m.p. > 230°, $[\alpha]_D^{20}-11.7°$ (c = 1, DMF), $R_f(2)-0.3$, $R_f(12)-0.3$, $R_f(26)-0.4$, $Lys/Orn_{1.93}Arg_{1.04}Asp_{2.03}Gly_{1.01}Ala_{1.94}Val_{0.95}Nle_{0.98}$, (Found: C, 60.63; H, 7.03; N, 12.09; $C_{138}H_{185}N_{23}O_{27}S.5H_2O$ requires: C, 60.93; H, 7.22; N, 11.84%).

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REFERENCES

The compound numbering sequence follows that established in earlier papers in this series.

[1]G. W. Kenner, *Proc. Roy. Soc. Lond. A.* **353**, 441 (1977).
[2]C. C. F. Blake and I. D. A. Swan, *Nature New Biol.* **232**, 12 (1971).
[3]I. J. Galpin, F. E. Hancock, B. K. Handa, A. G. Jackson, G. W. Kenner, R. Ramage and B. Singh, *Tetrahedron* **35**, 2771 (1979).
[4]R. Roeske, *J. Org. Chem.* **28**, 125 (1963).
[5]E. Wünsch and F. Drees, *Chem. Ber.* **99**, 110 (1966).
[6]G. Jäger and R. Geiger, *Ibid.* **103**, 1727 (1970).
[7]W. L. Hass, E. V. Krumkalns and K. Gerzon, *J. Am. Chem. Soc.* **88**, 1988 (1966).
[8]W. König and R. Geiger, *Chem. Ber.* **106**, 3626 (1973).
[9]J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara and K. Hofmann, *J. Am. Chem. Soc.* **93**, 5526 (1971).
[10]I. J. Galpin, P. M. Hardy, G. W. Kenner, J. R. McDermott, R. Ramage, J. H. Seely and R. G. Tyson, *Tetrahedron* **35**, 2577 (1979).
[11]I. J. Galpin, B. K. Handa, G. W. Kenner, S. R. Ohlsen and R. Ramage, *Ibid.* **36**, 2247 (1980).
[12]J. H. Sen, R. R. Semby and F. M. Bumpus, *J. Am. Chem. Soc.* **84**, 4948 (1962).
[13]M. Zaoral, *Coll. Czech. Chem. Comm.* **27**, 1273 (1962).